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Experimental African HEV Infection in Cynomolgus Macaques (*Macaca fascicularis*)

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Experimental infection with hepatitis E virus (HEV) from Africa has not been investigated. Our purpose was to study hepatitis E produced by HEV from Chad (North Africa) and to analyze the genetic sequence of the HEV obtained after animal passage. An HEV-containing fecal sample from Chad was intravenously inoculated in four cynomolgus macaques. When serum Alanine Amino Transferase (ALT) levels rose, open liver biopsy and bile aspiration were performed. In all the monkeys, an ALT rise occured 25 to 32 days after inoculation and new anti-HEV was detected by Enzyme Immuno Assay (EIA). Hepatic histopathology was consistent with acute viral hepatitis. HEV was detected by polymerase chain reaction (PCR) in bile (¾ animals) and feces (2⁄4 animals) and by imunoelectron microscopy (IEM) in the inoculum and one bile specimen. A genetic variant HEV was identified in one monkey. The Chad HEV produced hepatitis E with pathophysiologic and histopathologic findings similar to those observed with HEV from other geographic origins. A genomic variant HEV population was produced after one passage in a macaque. ***J. Med. Virol. 55:197–202, 1998.***

KEY WORDS: hepatitis E; Chad; HEV genomic variation; cynomolgus macaque

INTRODUCTION

Hepatitis E outbreaks have been reported in Mexico and several countries of Asia [Purcell, 1996; Ticehurst, 1995] and Africa [Coursaget et al., 1993, 1996; Mast et al., 1994; Molinié et al., 1986; Mushahwar et al., 1993; Tsega et al., 1991; van Cuyck-Gandré et al., 1996a, 1996b]. Experimental hepatitis E produced by HEV from Mexico [Ticehurst et al., 1992] and from Asian countries [Arankalle et al., 1995; Gupta et al., 1990; Jameel et al., 1992; Krawczinski et al., 1989; Tsarev et al., 1992; Vrati et al., 1992] has been extensively studied in monkeys. However, hepatitis E due to African HEV has been reported in only one monkey [Chatterjee et al., 1997].

An outbreak of hepatitis was described in French soldiers stationed in Chad (North Africa) in 1983 [Molinié et al., 1986] and serologically identified as hepatitis E [Coursaget et al., 1993]. HEV was subsequently detected by polymerase chain reaction (PCR) in the feces of patients in the outbreak [van Cuyck-Gandré et al., 1996a, 1996b]. The genome of an HEV isolate from a patient in the Chad outbreak has been partially sequenced [van Cuyck-Gandré et al., 1996b, 1997]. This Chad HEV is genetically more similar to the Asian strains than to the Mexican strain, but it represents a distinct phylogenetic group of HEV [van Cuyck-Gandré et al., 1997].

The purpose of the current study is to establish the infectivity of HEV in the 1983 Chad specimen, to document the course of hepatitis E produced by HEV from Africa, and to compare the genetic sequence of the HEV in the inoculum to the HEV shed by the macaques.

MATERIALS AND METHODS

Inoculum

An HEV-containing fecal sample collected from a patient (T3) with hepatitis E in the 1983 Chad outbreak was diluted in phosphate-buffered saline (PBS) (pH

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7.4) containing 1% bovine serum albumin (BSA) to make a 10% wt/vol suspension. The clarified suspension was subsequently filtered through 0.8-μm and 0.22-μm filters. HEV had been detected in the T3 fecal sample at 1:100 dilution by nested RT-PCR amplifying the ORF2 region of the genome [van Cuyck-Gandré et al., 1996a, 1996b). The clarified and filtered fecal suspension was screened for bacteria, yeast, and other viruses. Recommendations of the Guide for the Care and Use of Laboratory Animals of the National Research Council were followed; facilities for this research were fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, International. Animals were cared for by the Department of Animal Resources, WRAIR, according to biosafety level 2 precautions. The protocol was approved by the Laboratory Animal Care and Use Committee.

Inoculation Protocol

Animals. Four juvenile male (weight 5–9 Kg) wild-caught cynomolgus macaques (I4791, 554A, C168B, D421A) were inoculated intravenously with 1 cc of the inoculum.

Monitoring of monkeys. Preinoculation sera, feces, and liver biopsies were collected to establish a baseline for the parameters studied.

After inoculation, serial sera and feces were collected twice a week. The sera were tested for ALT the same day they were collected. When the serum ALT level exceeded the base line ALT mean plus two standard deviations in two consecutive serum specimens, an open liver biopsy, and bile aspiration were performed.

Analysis of Specimens

Sera were assessed for ALT levels and for anti-HEV antibodies. Liver biopsies were evaluated for histopathology by light microscopy. Feces and bile were tested for HEV by PCR. The genome of HEV detected in bile specimens was sequenced. The inoculum and one bile specimen were examined by imunoelectron microscopy (IEM) for HEV.

The sera were tested for anti-HEV IgM and IgG using an ELISA test based on the Sar-55 strain (Pakistan-Sargodha) ORF2 protein [Drabick et al., in press; Tsarev et al., 1993a, 1993b]. Preinoculation baseline sera were used for negative controls for each monkey.

HEV was detected by affinity capture followed by nested RT-PCR using two sets of nested primers selected in the ORF2 region of the genome as previously described [van Cuyck-Gandré et al., 1996a, 1996b]. In addition, from each PCR positive bile specimen a 504-nt fragment (nt 6095 to 6599) from the ORF2 region of the HEV was amplified by affinity capture and single-round RT-PCR and sequenced on both DNA strands for each PCR product [van Cuyck-Gandré et al., 1996b, 1997].

Immunoelectron Microscopy

IEM was also used to detect HEV in feces and bile. The T3 inoculum or aspirated bile from cyno 554A was clarified by centrifugation, and mixed with chimpanzee 1313 hyperimmune anti-HEV serum [Tsarev et al., 1993a, 1993b]. The chimpanzee serum had no HEV detectable by PCR. For controls, samples were incubated with preinoculation chimpanzee 1313 serum. After incubation, the specimens were placed on formvar–carbon-coated grids [Kelen et al., 1971] and stained with 2% phosphotungstic acid; grids were examined with a Zeiss electron microscope.

Sequence Analysis

The clustal alignment method from the DNA star program (DNASTAR Inc, Madison, WI) was used to compare the nucleic acid-deduced amino acid sequences to the sequence of the Chad HEV inoculum, and to the Burma [Tam et al., 1991] and Mexico [Huang et al., 1992] sequences. The computation was performed at the National Center for Biotechnology Information (NCBI) using the BLAST network service, and GenBank program [Altschul et al., 1990; Gish and States, 1993].

RESULTS

Serum ALT and Serology

All four HEV-inoculated macaques developed hepatitis based on ALT elevation. The ALT peaks occurred between 25 and 38 days after inoculation. The highest peak values were obtained for monkeys 554A and I4791, 274 and 404 respectively.

In the preinoculation sera, no anti-HEV IgM or IgG was detectable by EIA. Postinoculation, all four monkeys developed anti-HEV IgM and IgG. IgM was first detected between 17 and 24 days postinoculation, prior to the onset of the ALT rise. The IgM had return to baseline by 50 days postinoculation. The IgM rise was followed closely by the rise in anti-HEV IgG; the IgG remained markedly elevated three months after inoculation.

Histopathology

Preinoculation biopsies were normal. Open liver biopsy and bile aspiration were performed just after the ALT peak.

Following inoculation, Cyno C168B had normal liver histology (Fig. 1A) except for increased eosinophilia of some periportal hepatocytes. The three remaining monkeys had histopathologic changes. All three had proliferation of portal bile ducts and lobular inflammation with macrophages and small lymphocytes (Fig. 1B, 1C, 1D). D421A and I4791 had acidophilic bodies and cells in mitosis, indicating single-cell necrosis and regeneration of hepatocytes, respectively, as well as inflammation involving one of the central veins (Fig. 1B, 1D). In 554A, an increased amount of fat was present in mid-lobular and centrilobular hepatocytes (Fig. 1C).

The most prominent pathologies were observed in I4791. In some hepatocytes, apoptotic changes were ob-

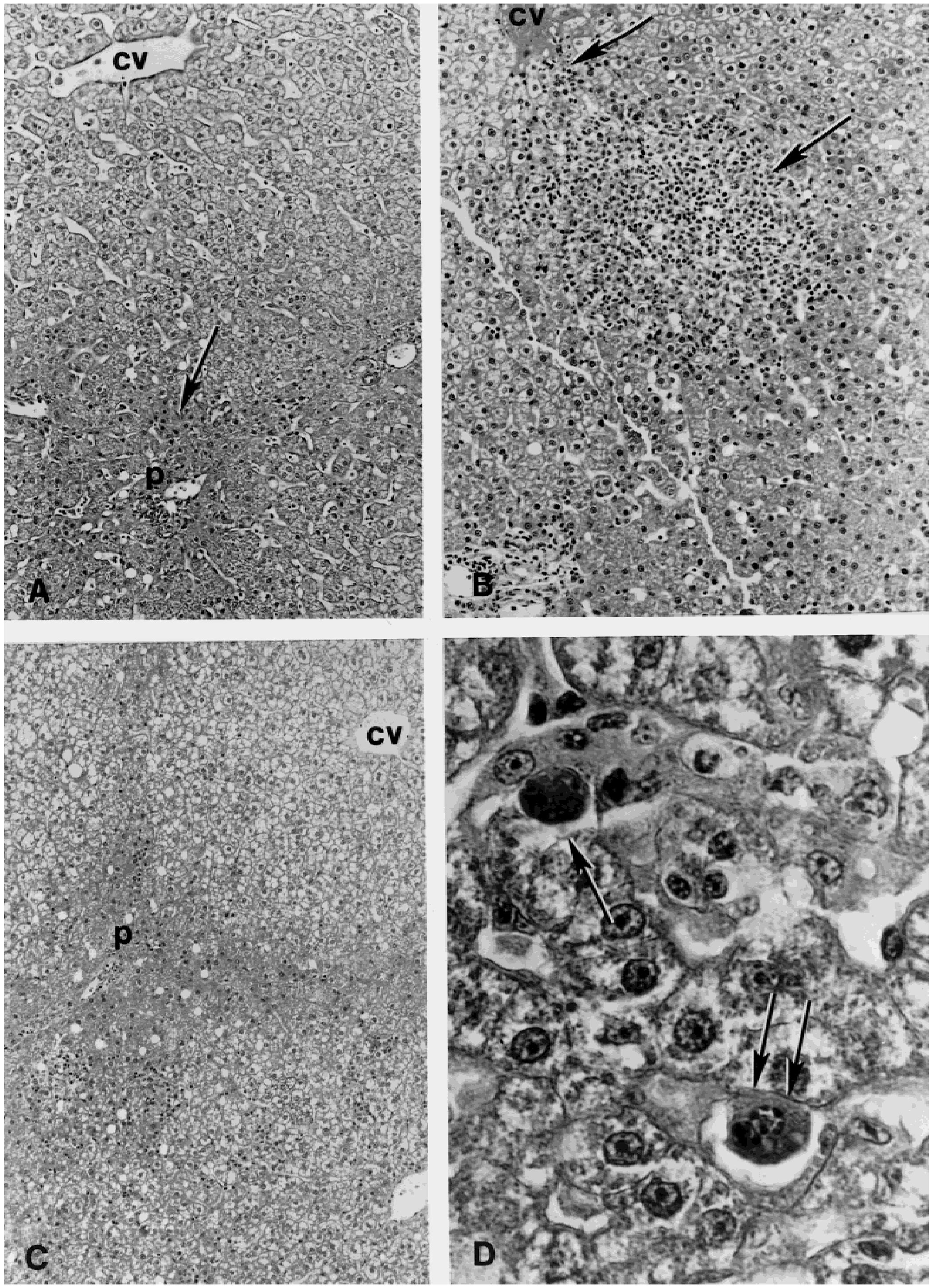

Fig. 1. Histopathological changes in the livers of cynomolgus monkeys (cynos) inoculated with Chad strain of HEV. **A:** Cyno C168B. Liver shows normal histology with slightly increased eosinophilia of periportal hepatocytes (arrow). Original magnification ×110. **B:** Cyno D421A. Lobular infiltrate consisting of lymphocytes and macrophages extends into vascular wall of the central vein (arrows). Original magnification ×150. **C:** Cyno 554A. Midlobular and centrilobular hepatocytes have increased amount of fat. Original magnification ×110. **D:** Cyno I4791. Single acidophilic body (arrow) and a hepatocyte showing apoptic changes in the nuclei (double arrow). Original magnification ×900. CV denotes central vein; P, portal area.

served in the nuclei, with margination and clumping of nuclear chromatin and condensation and increased eosinophilia of cytoplasm. Kupffer cells had prominent hypertrophy and hyperplasia (Fig. 1D).

Virus Shedding and Sequencing

HEV was detected in the bile of three monkeys (I4791, 554A, D421A) and in the feces of two monkeys

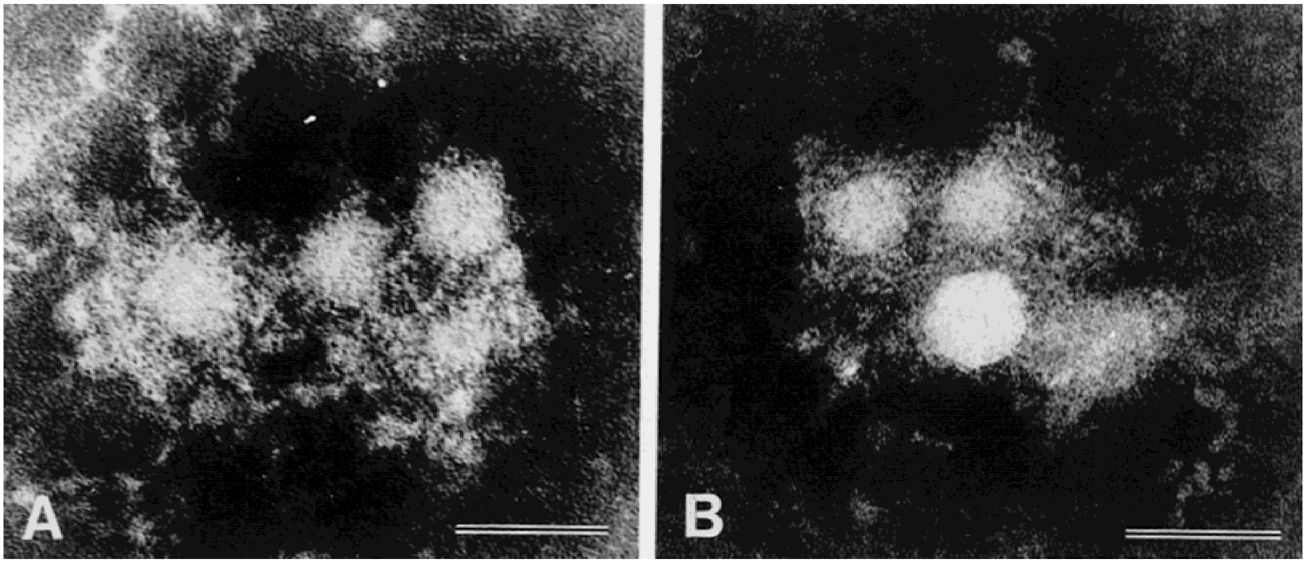

Fig. 2. HEV particles detected by immunoelectron microscopy in patient T3 feces **(A)** and in bile of a cynomolgus macaque **(B)** inoculated with T3 feces. Particles are surrounded by a fuzzy halo of antibodies and vary in size from 25 to 32 nm. Bar = 50 nm.

(I4791, C168B) during the second week postinoculation by RT-PCR.

IEM detected virus-like particles in the T3 inoculum (Fig. 2A) and in the bile of I4791 (Fig. 2B). In both the inoculum and the bile, aggregated 26-nm particles (average size) coated with antibodies were observed. No aggregated particles were observed in control preparations.

PCR products from the bile from I4791, 554A, and D421A were sequenced. A 128 amino acid nucleic acid deduced sequence, representing 384 nucleic acids of ORF2 (nt 6147 to 6531 of the Burma sequence) was compared to the sequence of the T3 inoculum and two reference strains (Burma and Mexico). All the HEV isolates from the macaques had the same Chad-specific peptide change: serine/threonine at position 23 of the sequence analyzed (Fig. 3), and the Mexico-like mutations at positions 92 and 102, previously shown as characteristic of the Chad isolate [van Cuyck-Gandré et al., 1997]. I4791 had an additional change, glutamic acid/glycine, at position 30. Peptide sequences from D421A and 554A were identical to the inoculum sequence.

DISCUSSION

All four macaques were infected by the Chad HEV and developed typical experimental hepatitis E. Based on standard screening procedures of the inoculum, no agent other than HEV was detected to contribute to the hepatitis.

The hepatic pathological changes observed in liver biopsies of three of the macaques indicated different stages of acute viral hepatitis. Similar findings including hepatocyte necrosis, inflammatory cell and lymphocyte infiltration, Kupffer cell hypertrophy, and single-cell necrosis have been described in other studies of experimental hepatitis E [Bradley et al., 1987, Andzhaparidze et al., 1986; Krawczynski et al., 1989; Longer et al., 1993; Soe et al., 1989; Ticehurst et al., 1992; Tsarev et al., 1993a, 1993b]. The findings were consistent with, but no specific to hepatitis E. The observation of increased hepatocyte fat (554A) was nonspecific but has been reported in other studies [Longer et al., 1993; Ticehurst et al., 1992]. Similarly, the significance of hepatocyte eosinophylia (C168B) is unclear but has been observed previously in macaques during HEV infection (unpublished data).

The sequence of pathophysiologic events was characteristic of experimental hepatitis E in macaques after intravenous inoculation. The earliest finding, during the second week of postinoculation, was the presence of HEV in the feces. By the start of the fourth week, all the animals had developed anti-HEV IgM. By the fifth week, all animals had ALT elevation, anti-HEV IgG, and three of four had hepatic histopathologic changes. These findings, including the variable severity of hepatitis, are in close accordance with previous studies using Sar55 [Tsarev et al., 1993a, 1993b] and Mexico-14 [Longer et al., 1993; Ticehurst et al., 1992]. The early presence of HEV replication without apparent hepatitis, the later appearance of anti-HEV followed closely by the onset of hepatitis, and the predominantly lymphocytic infiltrate in the liver in this study and others all suggest that hepatitis E is immune-mediated and not due to the direct effect of replicating HEV.

Comparison of the peptide sequence from the ORF2 portion of the genome of the HEV passed in the macaques to that of the inoculum and two references strains, Mexico and Burma, showed that variation in the sequence can occur after a single animal passage of HEV. Similar findings have been described for hepatitis A virus, an enterically transmitted RNA virus, after passage in animals or cell culture [Cohen et al., 1989; Emerson et al., 1992; Lemon et al., 1987]. Otherwise,

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                   10                  20                  30                  40                  50
1    G A D G T A E L T T T A A T R F M K D L Y F S S T N G V G E I G R G I A L T L F N L A D T L L G G L   D421A-cyno
1    G A D G T A E L T T T A A T R F M K D L Y F S S T N G V G E I G R G I A L T L F N L A D T L L G G L   554A-cyno
1    G A D G T A E L T T T A A T R F M K D L Y F S S T N G V G G I G R G I A L T L F N L A D T L L G G L   I4791-cyno
1    G A D G T A E L T T T A A T R F M K D L Y F S S T N G V G E I G R G I A L T L F N L A D T L L G G L   T3-human
1    G A D G T A E L T T T A A T R F M K D L Y F T S T N G V G E I G R G I A L T L F N L A D T L L G G L   HPESVP/Burma
1    G A D G T A E L T T T A A T R F M K D L H F T G L N G V G E V G R G I A L T L L N L A D T L L G G L   HPENSSP/Mexico

                   60                  70                  80                  90                  100
34   P T E L I S S A G G Q L F Y S R P V V S A N G E P T V K L Y T S V E N A Q Q D K G I A I P H D I D L   D421A-cyno
34   P T E L I S S A G G Q L F Y S R P V V S A N G E P T V K L Y T S V E N A Q Q D K G I A I P H D I D L   554A-cyno
34   P T E L I S S A G G Q L F Y S R P V V S A N G E P T V K L Y T S V E N A Q Q D K G I A I P H D I D L   I4791-cyno
34   P T E L I S S A G G Q L F Y S R P V V S A N G E P T V K L Y T S V E N A Q Q D K G I A I P H D I D L   T3-human
51   P T E L I S S A G G Q L F Y S R P V V S A N G E P T V K L Y T S V E N A Q Q D K G V A I P H D I D L   HPESVP/Burma
50   P T E L I S S A G G Q L F Y S R P V V S A N G E P T V K L Y T S V E N A Q Q D K G I A I P H D I D L   HPENSSP/Mexico

                 110                 120
84   G E S R V V I Q D Y D N Q H E Q D R P T P S P A P S R P   D421A-cyno
84   G E S R V V I Q D Y D N Q H E Q D R P T P S P A P S R P   554A-cyno
84   G E S R V V I Q D Y D N Q H E Q D R P T P S P A P S R P   I4791-cyno
84   G E S R V V I Q D Y D N Q H E Q D R P T P S P A P S R P   T3-human
101  G D S R V V I Q D Y D N Q H E Q D R P T P S P A P S R P   HPESVP/Burma
100  G E S R V V I Q D Y D N Q H E Q D R P T P S P A P S R P   HPENSSP/Mexico
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Fig. 3. Alignment of the nucleic acid-deduced sequences in the ORF2 region of the HEV genome from monkey biles (D421A cyno, 554A cyno, and I4791 cyno), from the inoculum (T3 human), and from two reference strains, HPESVP/Burma and HPENSSP/Mexico.

the amino acid sequence specific to the Chad inoculum was conserved in all cynos. Interestingly, this variant was isolated from the macaque with the most severe hepatitis.

This study demonstrates the infectivity of the Chad HEV in cynomolgus macaques and documents the similarity of experimental hepatitis E regardless of the geographic origin or sequence differences of the infecting strains of HEV. Genetic variants of HEV arising during animal infection indicate that multiple HEV subpopulations may appear. Whether the specific variants that arise influence the course or severity of the hepatitis remains unknown.

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